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Development of a Novel Mouse Model for Dengue Virus Infection

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In the present study, we established an animal model for dengue virus infection using severe combined immunodeficient mice transplanted with a human hepatocarcinoma cell line (HepG2). At 7–8 weeks after transplantation, the HepG2-grafted mice were infected intraperitoneally with dengue virus type 2 (DEN-2). A higher titer of the virus was detected in the liver and serum but not in the brain in the early stage of postinfection. When the mice showed paralysis, the highest titer of virus was detected in the serum and brain. DEN-2 antigens were also found in HepG2 cells of the liver in the early stage and some neurons of the brain in the late stage. Upon clinical examination, thrombocytopenia, prolonged partial thromboplastin time, and increased hematocrit, blood urea nitrogen, and tumor necrosis factor α were seen in the paralyzed mice. Moreover, mild hemorrhage in the liver and tarry stool in the small intestine were observed in some mice. Our results show some similarities to human DEN infection and this mouse model might be valuable for studying some aspects of pathogenesis of this disease.

INTRODUCTION

Dengue (DEN) viruses are mosquito-borne RNA viruses that are classified serologically into four antigenically distinct types (DEN-1, 2, 3, 4). They infect millions of people in tropical and subtropical regions of the world and can cause a mild to debilitating febrile illness (classical dengue fever, DF) or life-threatening syndrome (dengue hemorrhagic fever/dengue shock syndrome, DHF/DSS). However, the pathogenesis of human dengue infection remains unknown and no successful vaccine is available as yet (Eckels, 1993). A major difficulty in studying dengue infection and developing a dengue virus vaccine is the absence of a suitable animal model (Bhamarapravati, 1993). Experimental infection of rhesus monkeys results only in low-titer viremia of short duration (Innis *et al.,* 1988). The availability of monkeys for experimentation is very scarce due to wildlife conservation policies. Severe combined immunodeficient (SCID) mice do not produce functional T and B cells and lack detectable immunoglobulin (Bosma *et al.,* 1983). SCID mice reconstituted with human peripheral blood lymphocytes and infected with DEN-1 virus were first used to develop an animal model (Wu *et al.,* 1995). However, only some of these mice showed sensitivity to DEN-1 infection.

In the past few years, unusual clinical manifestations, mostly cerebral and hepatic symptoms, have become more common in patients with DEN virus infection (Rajajee and Mukundan, 1994; Thisyakorn and Thisyakorn, 1994). Although the liver is not a major target organ, the involvement of liver cells in pathogenesis of DEN virus infection has been indicated by the abnormal liver function, pathological findings, and detection of viral antigen in hepatocytes and Kupffer's cells at biopsies (Bhamarapravati *et al.,* 1967; Burke, 1968; Rosen *et al.,* 1989; Kuo *et al.,* 1992). It was reported recently that DEN virus can replicate in a human hepatocarcinoma cell line, HepG2, and infectious particles are released into the culture medium (Marianneau *et al.,* 1996; 1997). We have transplanted HepG2 cells into SCID mice to develop an animal model for studying the pathogenesis of DEN virus infection.

RESULTS

Serum hALB levels and replication of HepG2 cells in host mice

The transplanted HepG2 cells grew in the grafted mice. Serum hALB, which is released from transplanted HepG2 cells, could not be detected in host mice until 14 days after transplantation. The level then increased and peaked at day 60 followed by a gradual decrease (Fig. 1). In gross and microscopic observations, proliferating HepG2 cells were usually distributed in the liver (arrows in Fig. 2) and less frequently in the spleen. However, HepG2 cells were not observed in the lung, small intestine, or brain. These findings show that HepG2 cells

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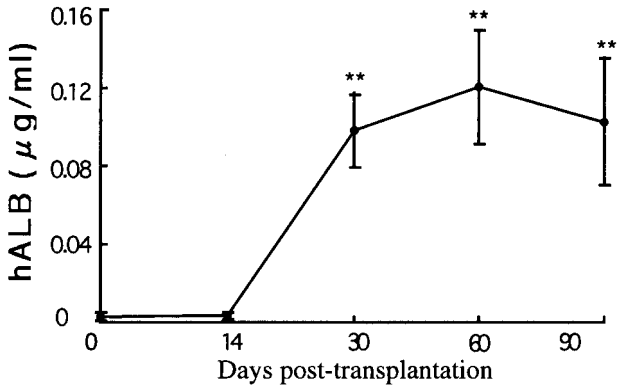

FIG. 1. Level of human albumin in the serum of SCID mice after transplantation of HepG2 cells. Values represent mean ± SD, $n = 5$ animals. $^{**}P < 0.01$ vs before HepG2 cell transplantation.

injected through the spleen translocated into the liver of host mice and propagated there. The rate of successful transplantation was about 90% in HepG2-inoculated mice ($n = 20$) and the mice were healthy and survived more than 12 weeks after transplantation.

DEN-2 virus infection of HepG2-grafted mice; Clinical signs

After intraperitoneal (ip) infection with DEN-2 virus, the HepG2-grafted SCID mice gradually developed anorexia, asthenia, and decreased body weight. At day 13 to 18 postinfection (p.i.), all of these mice showed hind-leg paralysis and lower body temperature. Biopsies showed that the liver usually shrank or occasionally became slightly enlarged, and most of the HepG2 cells were destroyed by DEN virus indicated by gross or histological view of livers in which HepG2 cell masses disappeared. Small quantities of ascites were observed in about 25% of the infected HepG2-grafted mice at day 11 p.i. and the day of paralysis onset. Tarry stool in the small intestine was sometimes seen in the infected, grafted mice but not in the control mice. Most of the infected, grafted mice (about 85%) died 6–12 h after onset of paralysis.

Clinical parameters of the HepG2-grafted mice with ip infection are summarized in Table 1. PC gradually decreased from day 5 p.i. and was significantly lower than that of uninfected HepG2-grafted mice at day 8 p.i. ($P < 0.05$). The lowest platelet count (PC) (40×10^4 platelets/mm^3) was seen at day 13–18 p.i. (paralysis onset; DP in Table 1), and it was significantly different from those of days 5, 8, and 11 p.i. and the control group ($P < 0.01$). Partial thromboplastin time (PTT) was prolonged and hematocrit (Ht) was increased in all of the paralyzed mice. Serum tumor necrosis factor α (TNF α) and blood urea nitrogen (BUN) did not change during the early stage of DEN-2 virus infection, but both dramatically increased in all of these mice at the onset of paralysis (DP, in Table 1). At that time, TNF α and BUN values were 718 pg/ml and 33 mg/dl, respectively, which were significantly higher than those of the control group ($P < 0.01$).

In contrast to infected, HepG2-grafted SCID mice, anorexia, asthenia, and decreased body weight were not seen in uninfected, HepG2-grafted SCID mice. Clinical parameters of PC, PTT, TNF α, and BUN in these mice were 80.39×10^4 platelets/mm^3, 10 s, 45.00 pg/ml, and 16.80 mg/dl, respectively. Compared with ungrafted mice without infection, they did not show any changes except for slightly increased Ht (Tables 1 and 2). Biopsy showed only replicating HepG2 cell masses in the liver of these mice and no ascites were seen. In the ip-infected, ungrafted SCID mice, although 15% of these mice ($n = 20$) had hind-leg paralysis 35–60 days p.i., neither abnormalities in gross and histological examinations of several organs (liver, lung, spleen, and small intestine) nor changes of PC, PTT, Ht, serum TNF α, and BUN were observed.

Distribution of DEN-2 virus in HepG2-grafted mice

Virus titers in the serum and several organs at different times after ip DEN-2 virus infection are shown in Fig. 3. Virus was detected in the livers of all of the infected, HepG2-grafted mice. Virus titer was $10^{4.1}$ PFU/g at day 5 p.i. and peaked at day 8 at about $10^{4.9}$ PFU/g. At day 11 and the day of paralysis onset (13–18 days p.i.), titers decreased, and virus was detected in only 60 and 40% of infected mice, respectively.

Viremia was detected in 60% of the mice at day 5 p.i. and in 80% of the mice at the day 8 p.i. with titers of $10^{3.2}$–$10^{6.0}$ PFU/ml. From day 11 p.i. to the onset of paralysis, viremia at $10^{3.8}$–$10^{5.7}$ PFU/ml was detected in all of the infected, HepG2-grafted mice. Virus was not detected in the brain until day 11 p.i., when virus was measured at $10^{3.3}$–$10^{5.6}$ PFU/g in 80% of the mice (Fig. 3). High virus titers of $10^{5.6}$–$10^{6.6}$ PFU/g were seen in all of these mice on the day of paralysis onset. Virus was not detectable in other organs, including spleen, lung, and small intestine throughout the course of disease. Moreover, when ungrafted SCID mice were inoculated ip with DEN-2 virus, there was no detectable virus in the serum or liver. However, virus ($10^{3.0}$ PFU/g) was detected in the brain of 15% these mice 35–60 days p.i.

Histopathological and immunohistochemical observations of DEN-2 virus-infected, HepG2-grafted mice

In hematoxylin and eosin (HE) staining of liver sections of DEN-2 virus-infected, HepG2-grafted mice, mild hemorrhage, congestion, and edema were seen in mice liver cell masses of some lobules and HepG2 cell masses (Fig. 4a). Hepatocyte swelling, cytoplasmic vacuolation, and hypertrophy of Kupffer's cells were also observed in the livers of the mice. In the small intestine, mucosal

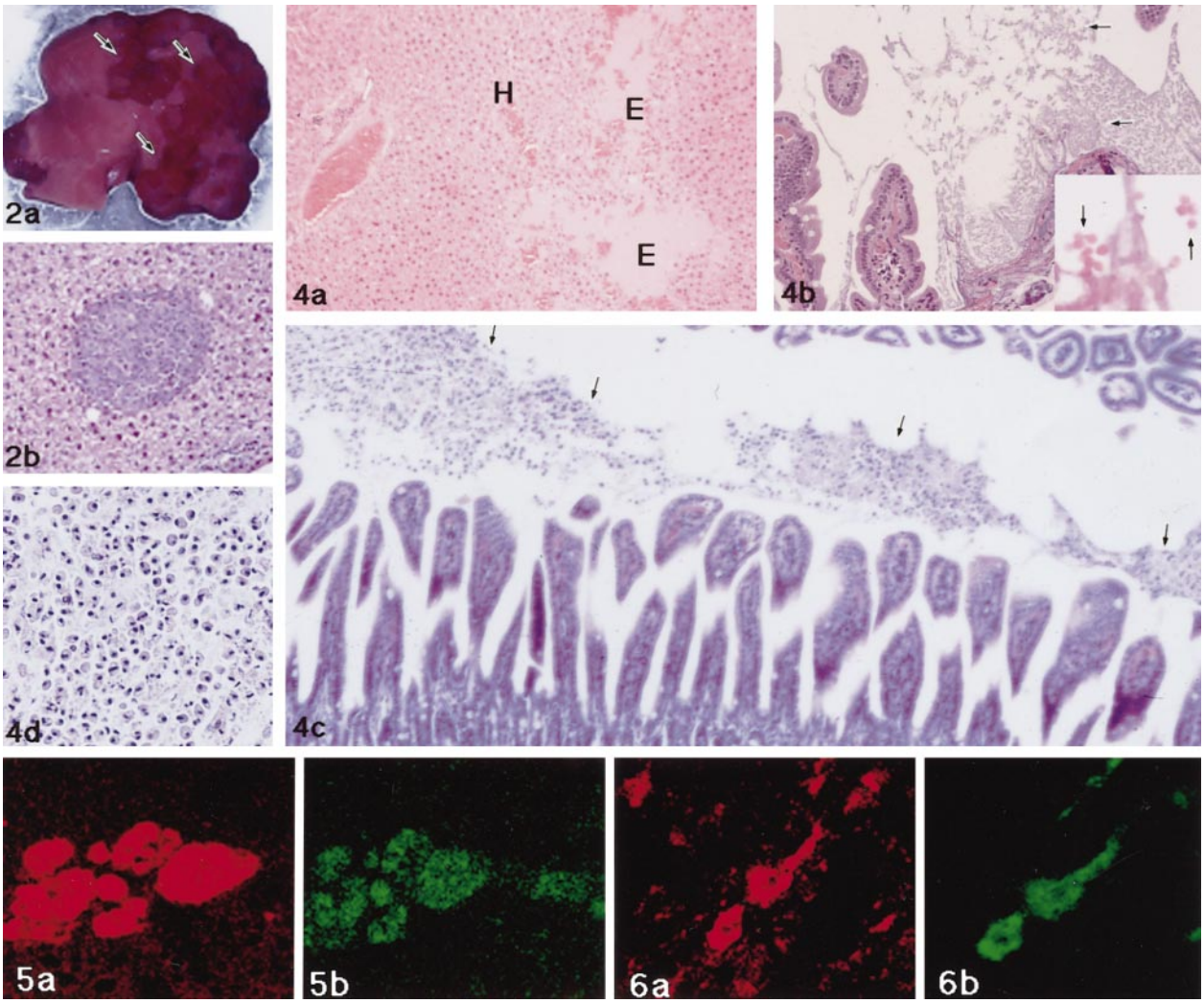

FIG. 2. Replication of HepG2 cells (arrows) in the SCID mouse liver at day 60 after transplantation. (a) Gross examination, ×3; (b) hematoxylin and eosin staining, ×100.

FIG. 4. Histopathological observation of organs from HepG2-grafted SCID mouse inoculated ip with DEN-2 virus. (a) Section of the liver at day 11 after infection had hemorrhage (H), congestion, and edema (E) in host liver cells of some lobules. HE staining, ×150. (b) Small quantity of fresh blood (arrows) in the mucosae of the small intestine at the day of paralysis onset. HE staining, ×200. (Inset) A higher magnification showing the red blood cells, ×400. (c) Macrophage infiltration (arrows) in the lumen of the small intestine of a paralyzed mouse, HE staining, ×100. (d) A higher magnification of the macrophages, ×300.

FIG. 5. Double-staining of liver sections to identify the site of DEN-2 virus replication in infected, HepG2-grafted SCID mice. (a) Section of the liver was stained with anti-human albumin (a marker for HepG2 cells) antibodies and (b) stained with anti-DEN monoclonal antibodies (a mixture of MAb 504 and 301) (Kimura-Kuroda and Yasui, 1986). DEN virus antigens were seen in HepG2 cells, ×350.

FIG. 6. Double-staining of brain sections to identify the site of DEN-2 virus replication in infected, HepG2-grafted SCID mice. (a) Section of the brain was stained with anti-microtubule-associated protein 2 (a marker for neurons) antibodies and (b) stained with anti-DEN monoclonal antibodies (Kimura-Kuroda and Yasui, 1986). DEN virus antigens were seen in neurons. ×350.

congestion, small quantities of fresh blood in the mucosae (Fig. 4b), and macrophage infiltration into the lumen of the intestine were observed in some paralyzed mice (Figs. 4c and 4d). No histopathological changes were seen in the liver and small intestine of control uninfected, HepG2-grafted mice or control virus-infected, ungrafted mice.

To determine whether DEN-2 virus replicated in the HepG2 cells or normal mouse hepatocytes, liver sections were stained for both DEN viral antigens and human albumin (hALB) by double staining. Viral antigens were detected in most of the hALB-positive HepG2 cells but not in the hepatocytes of the host mouse (Figs. 5a and 5b). Moreover, viral antigens were frequently observed in the neurons when the brain sections were double-stained with anti-microtubule-associated protein 2 (anti-MAP2) antibodies and anti-viral monoclonal antibodies 301/504 (Figs. 6a and 6b). No detectable viral antigens were seen in the lung, spleen, and small intestine.

TABLE 1

Changes of Platelet Count (PC), Partial Thromplastin Time (PTT), Hematocrit (Ht), Serum Tumor Necrosis Factor α (TNF α), and Blood Urea Nitrogen (BUN) after Intraperitoneal Infection of HepG2-Grafted Mice with Dengue Type 2 Virus

Days p.i.	PC ($\times 10^4/\mu$l)	PTT (s)	Ht (%)	TNF α (pg/ml)	BUN (mg/dl)
5	70.28 ± 11.43	11.00 ± 1.0	46.50 ± 4.51	22.10 ± 49.19	17.31 ± 1.35
8	63.82 ± 8.48*	11.40 ± 1.29	48.00 ± 3.54	55.00 ± 51.71	19.58 ± 2.85
11	48.53 ± 17.86**	11.30 ± 0.45	49.60 ± 2.07	61.00 ± 42.18	17.40 ± 3.37
DP[a]	40.30 ± 15.34**	29.35 ± 11.98**	55.20 ± 1.64**	718.33 ± 215.17**	33.31 ± 14.09**
Cont[b]	80.39 ± 11.92	10.21 ± 1.10	45.80 ± 2.59	45.00 ± 44.72	16.80 ± 1.86

Note. Each average value is from seven mice.
[a] DP, day of paralysis onset (range 13–18 days postinfection).
[b] Cont, control group (HepG2-grafted mice without infection).
* $P < 0.05$ vs Cont.
** $P < 0.01$ vs Cont.

Comparison of clinical parameters and virus titers between HepG2-grafted SCID mice infected ip and ungrafted, SCID mice infected intracerebrally (IC)

Ungrafted SCID mice infected ic with DEN-2 virus also showed hind-leg paralysis at day 12 p.i. and then died at day 13 or 14 p.i., although biopsy showed no significant abnormal findings. These mice were examined for the levels of PC, PTT, Ht, TNF α, and BUN and the data were compared with those of the HepG2-grafted mice infected ip (Table 2). On the day of paralysis onset, the ic group showed no abnormal changes in PTT, Ht, TNF α, and BUN and only slightly decreased PC. In contrast, the ip group showed drastically decreased PC, prolonged PTT, and increased Ht, TNF α, and BUN. The difference between two groups for each parameter was significantly ($P < 0.01$).

Virus distributions in the mice of both groups were also compared (Table 2). Although equivalent virus titers, $10^{5.7}$ PFU/g, were detected in the brains of both groups, only a few mice in the ic group had a low titer of virus in the serum, and no virus was detected in the liver of these mice. On the other hand, virus was usually detected in the serum (average of $10^{4.7}$ PFU/ml) and liver ($10^{3.9}$ PFU/g) of all of the HepG2-grafted mice in the ip group ($P < 0.01$ for the serum).

DISCUSSION

HepG2-grafted SCID mice had a high sensitivity to DEN virus infection

To develop a useful animal model for DEN virus infection, HepG2 cells, which support DEN viral replication (Marianneau *et al.,* 1996, 1997), were transplanted into SCID mice. Replication of HepG2 cells in the host mice was confirmed by an increase of serum hALB and propagation of HepG2 cells in the liver. These results indicated that HepG2 cells injected into the spleen migrated to the liver and grew there. The successful transplantation rate via the spleen was more than 90% and was higher than other transplantation routes (about 40% success), such as intraperitoneal injection or direct injection into the liver (data not shown).

All of these virus-infected mice showed gradual illness ending in paralysis and cachexy, and finally death. From

TABLE 2

Comparison of Virus Titer and Clinical Parameters between Mice Infected ic or ip

	ic infection ungrafted mice	ip infection HepG2-grafted mice	Ungrafted mice without infection
Virus titer (log PFU/g)			
Serum	1.280 ± 1.420	4.656 ± 0.687**	
Brain	5.732 ± 0.256	5.666 ± 0.417	
Liver	ND	3.941 ± 0.334	
PC ($\times 10^4/\mu$l)	55.61 ± 15.58	40.30 ± 15.34**	77.59 ± 15.31
PTT (s)	12.63 ± 1.11	29.35 ± 11.18**	11.43 ± 1.21
TNF α (pg/ml)	12.50 ± 25.00	718.33 ± 215.17**	10.32 ± 23.40
BUN (mg/dl)	17.13 ± 3.87	33.31 ± 14.09**	20.32 ± 4.34
Ht (%)	40.57 ± 2.53	55.20 ± 1.64**	38.94 ± 2.31

Note. ND, not detectable. Each average value is from seven mice killed at the day of paralysis onset.
** $P < 0.01$ vs ic infection.

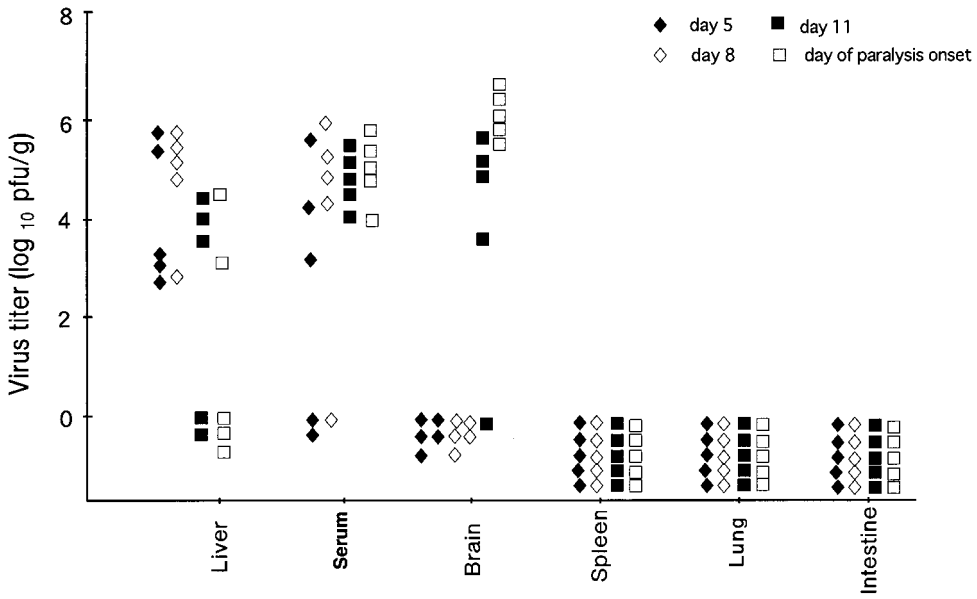

FIG. 3. Distribution of DEN-2 virus in the serum and several organs of HepG2-grafted SCID mice infected intraperitoneally with DEN-2 virus. Virus titer was determined by plaque assay on a Vero cell monolayer culture. Each point represents one sample from one mouse.

day 5 to the day of paralysis onset, the frequency of viremia increased from 60 to 100%, and the virus titers increased. In contrast, when ungrafted SCID mice were inoculated ip with DEN-2 virus, neither detectable viremia nor significant clinical manifestations were observed in most of mice. These results indicated that HepG2-grafted SCID mice had a higher sensitivity to DEN virus infection, and the transplantation of HepG2 cells was important in achieving this high sensitivity.

It is interesting that higher titers of virus were seen in the livers of all mice at the early stage p.i., but the titers decreased gradually. This might have resulted from exhaustion of HepG2 cells due to viral replication. On the other hand, both frequency and titer of viremia were low at the early stage, but increased gradually and reached the highest level at the day of paralysis onset. Virus titer in the brain also increased from day 11 to the day of paralysis onset. These results strongly suggested that DEN-2 virus replicated in the liver at first and then disseminated to the brain through the bloodstream, showing a disease course somewhat similar to that of human DEN virus infection in which virus first replicated in local tissue after infection and then was carried via the bloodstream into brain (Lum *et al.,* 1996; Nimmannitya *et al.,* 1987).

Target cells and organs for DEN virus replication in humans remain unclear. Mononuclear phagocytes have been considered as only one site of virus replication in humans (Halstead, 1981). In the present study, high virus titer and viral antigens were detected in the transplanted HepG2 cells in the liver. This is consistent with results from Rosen *et al.* (1989), who reported that recovery of the virus from the liver was very common in human DEN virus infection. High virus titer and viral antigens were also observed in the brain of infected mice. Those results indicated that HepG2 cells and neurons are main sites of viral replication of these mice. Recently, we found that DEN virus replicated in neurons of the anterior horn of the lumbar spinal cords in paralyzed mice (unpublished data), which might partially explain the occurrence of hind-leg paralysis.

DHF/DSS-like manifestations were observed in our model mice

It has been reported that thrombocytopenia and prolonged PTT are common in a severe form of human DEN virus infection. PTT and PC are usually used as potential indices for predicting DHF (Chua *et al.,* 1993; Hathirat *et al.,* 1993; Garcia *et al.,* 1995). In this study, the infected, HepG2-grafted SCID mice showed gradual decreases in PC and prolonged PTT. Moreover, mild hemorrhage in the liver and small intestine was also seen. In human DHF, gastrointestinal bleeding and small focal hemorrhages in the liver are very common (Burke, 1968; Rosen *et al.,* 1989; Rajajee and Mukundan, 1994). It seems that the mice showed some DHF-like manifestations.

BUN is a final metabolic product of protein and is eliminated from the kidney. In general, an elevated BUN level suggested renal dysfunction, hemoconcentration, or gastrointestinal bleeding. In the present study, increased Ht and BUN were seen in the paralyzed mice and small quantities of ascites were seen in some mice, indicating that the infected mice had some degree of hemoconcentration and renal dysfunction. It is probable that these resulted from increased vascular permeability and extravasation of fluid into interstitial spaces. It might be inferred that the mice showed evidence of shock.

However, some disease presentations in infected, HepG2-grafted SCID mice were different from that of human DEN infection. For example, viremia is usually no longer detectable at the time of onset of DHF/DSS in humans (Vaughn *et al.,* 1997), whereas DHF/DSS-like manifestations were seen at the late stage when viremia showed high titer in this animal model. It may be related to the fact that SCID mice do not produce specific antibodies to neutralize the virus. The differences might make some limitations of use of this animal model to extrapolate the situation in humans. Nevertheless, the animal model was considered to be helpful for studying some aspects of human DEN infection.

Although the mechanism of hemorrhage in DHF is unclear, decreased PC is considered to play a major role (Isarangkura and Tuchinda, 1993). The pathogenesis of thrombocytopenia is poorly understood. La Russa and Innis (1995) suggested that DEN virus-induced bone marrow suppression resulted in thrombocytopenia through depressed platelet synthesis in human DHF. Wang *et al.* (1995) proposed the possibility that binding of DEN virus to platelets might be involved in the thrombocytopenia in DHF. In the present study, in addition to thrombocytopenia, prolonged PTT was seen. The findings indicate that not only thrombocytopenia, but also consumptive coagulopathy played a critical role in the bleeding tendency in the mice. Further studies are needed to clarify the underlying mechanism of hemorrhage in human DEN infection.

TNF α might be involved in the etiology of shock in DHF/DSS, since TNF α is a strong inflammatory mediator and is released from macrophages activated by acute viral infection, endotoxin, or phagocytosis. In the present study, the infected mice showed increased TNF α in the serum and macrophage infiltration into the lumen of the small intestine as well as hypertrophy of Kupffer's cells in the liver. Macrophages activated by DEN-2 virus infection might be an important source of TNF α. Dysfunction or destruction of vascular endothelial cells by TNF α would cause increased vascular permeability leading to some degree of shock (Kurane and Ennis, 1994, 1997). Recently, significant elevation in serum TNF α has been reported in patients with DHF/DSS, and a close relationship between sequential changes in serum TNF α level and clinical progression of the disease has been shown (Yadav *et al.,* 1991; lyngkaran *et al.,* 1995). Exposure of vascular endothelial cells to TNF α dramatically increased adhesive interaction of the cells with neutrophils *in vitro* (Anderson *et al.,* 1997). It is likely that TNF α participated in the etiology of some DHF/DSS-like manifestations observed in the mice in this study.

Finally, the above-described various DHF/DSS-like manifestations in HepG2-grafted mice infected ip with DEN-2 virus are not directly associated with viral replication in the brain, because ungrafted-SCID mice infected ic showed none of these DHF/DSS-like findings, although the latter mice showed hind-leg paralysis and viral replication in the brain. Our mouse model has several clinical, hematological, histopathological, and virological similarities to human DEN infection. This appears to be a valuable mouse model for studying some aspects of pathogenesis of the disease.

MATERIALS AND METHODS

Mice

Female SCID (C.B.-17/Icr Tac-scid) mice were purchased from Clea Co. (Tokyo, Japan). They were maintained in sterile cages in a specific pathogen-free room and were given sterile water and food.

Transplantation

HepG2 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS), 4 mM glutamine, penicillin, and streptomycin at 37°C. The cells were dissociated with 0.25% trypsin and then suspended at 1.5×10^8 cells/ml in Haemaccel (Hoechst-Russel Pharmaceuticals) containing 50% FBS. These HepG2 cells were injected in a volume of 60–70 μl (about 1×10^7 cells) with a 26-gauge needle into the spleen of each ether-anesthetized SCID mouse aged 8 weeks. To confirm successful transplantation, the concentration of hALB in the serum was determined by an enzyme-linked immunosorbent assay (ELISA), and the liver and spleen were taken for histological observation at time intervals after transplantation.

Virus inoculum

DEN-2 (strain Tr1751) virus, which was isolated from a patient with DF and kindly provided by Dr. Ohtani A (National Institute of Infectious Diseases, Japan), was propagated in *Aedes albopictus* mosquito (C6/36) cells (Igarashi, 1978) and stored at −80°C until use. The titer of virus stock was adjusted to 2×10^6 PFU/ml.

Virus infection

The design of the experiment is shown in Table 3. HepG2-grafted SCID mice were infected ip with 10^6 PFU/mouse of DEN-2 virus at 7–8 weeks after transplantation. As controls, the following three experimental groups were prepared: HepG2-grafted SCID mice aged 15–16 weeks, without infection; ungrafted SCID mice aged 16 weeks, ip infection with 10^6 PFU/mouse of DEN-2 virus; ungrafted SCID mice aged 16 weeks, intracerebral inoculation with 10^6 PFU/mouse of DEN-2 virus.

Clinical parameters

The infected mice were observed daily for signs of morbidity, especially visible hemorrhage, tarry stool, and paralysis. Rectal body temperature was measured. Un-

TABLE 3

Design of the Experiment

Groups	Transplantation[a]	DEN-2 virus inoculation		Time of sacrifice
		Mice age[b]	Dosage and route	
1	+	16 weeks	10^6 PFU/mouse ip	Days 5, 8, and 11 and at paralysis onset
2	+	16 weeks	No infection	10 weeks posttransplantation
3	−	16 weeks	10^6 PFU/mouse ip	Day of paralysis onset
4	−	16 weeks	10^6 PFU/mouse ic	Day 12 postinfection (day of paralysis onset)

Note. ip, intraperitoneal; ic, intracerebral.

[a] Transplantation (+) or without transplantation (−).

[b] All mice had severe combined immunodeficiency (SCID).

der ether anesthesia, samples of blood were obtained from the heart and/or vascular plexus of brachium at days 5, 8, and 11 p.i. and at the day of paralysis onset. Samples of brain, liver, spleen, lung, and small intestine were taken at the same time. The blood sample was used for clinical examinations and virus titration as follows: (1) platelet count and hematocrit; (2) partial thromboplastin time was assayed using a thromboplastin test kit (Wako); (3) serum blood urea nitrogen was measured using a B-test kit (urease-indophenol method, Wako); (4) the concentration of TNF α in the serum was determined by ELISA using a mouse TNF α EIA Kit (PerSeptive Biosystems, USA). Each value was determined from the mean of two determinations; (5) virus titer in the serum was determined by a plaque assay as described below. The organ samples of each mouse were divided into two portions: one was immediately frozen at −80°C for titration of virus and the other was subjected to histopathological observation or immunostaining for DEN viral antigens.

Titration of virus

The organ samples were ground up in a mortar with Eagle's minimum essential medium supplemented with 20% Haemaccel, 10 mM HEPES (pH 7.2–7.4). The organ suspensions were centrifuged at 15,000 rev/min for 15 min at 4°C and then the supernatants were assayed for virus titer by the plaque method using Vero cell monolayer culture under methylcellulose overlay medium. Virus titer in the serum was also determined by the same plaque procedure.

Antibodies for immunostaining

Two mouse monoclonal antibodies, 504 and 301, were employed for detecting DEN viral antigens. Both monoclonal antibodies react with DEN viruses (Kimura-Kuroda and Yasui, 1986). Rabbit anti-hALB polyclonal antibody (Dako), which does not react with mouse albumin, was used as a marker for HepG2 cells. Rabbit anti-microtubule-associated protein 2 antibody, used as a marker of neurons, was kindly provided by Dr. Murofushi of Tokyo University.

Histopathological examination and immunostaining

The portions of organ samples were further divided into two parts. One was fixed with 10% formalin and processed for HE staining, and the other was cryosectioned for immunostaining to search for sites of DEN virus replication. In brief, liver or brain sections were incubated with DEN reactive monoclonal antibodies (mixture of 504 and 301) and anti-hALB or -MAP2 antibodies, respectively. After being washed, the sections were further incubated with biotinylated anti-mouse IgG (Amersham) and followed by Texas red-conjugated strevptoavidin (Amersham) or fluorescein isothiocyanate-conjugated anti-rabbit IgG (Dako). The specimens, including lung, spleen, and small intestine, were also stained for DEN viral antigens with the same procedure. The stained cells were observed by confocal laser microscopy (Molecular Dynamics, Sunnyvale, CA).

Statistics

Statistical analysis was conducted using Student's *t* test. *P* values <0.05 were considered significant.

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